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Accumulation of 10-kilobase DNA replication intermediates in cells treated with 3-aminobenzamide

[poly(ADP-ribose) synthetase/melanoma cells/DNA synthesis]

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ABSTRACT **During eukaryotic DNA synthesis there is formation of, in addition to Okazaki fragments, discrete 10-kilobase (kb) DNA replication intermediates. We have investigated the ligation of 10-kb DNA replication intermediates to high molecular weight DNA, using the drug 3-aminobenzamide, an inhibitor of poly(ADP-ribose) synthetase. In human melanoma cells treated with this inhibitor, there is an accumulation of 10-kb DNA. In contrast, in cells treated with aphidicolin, which inhibits DNA polymerase α, there is continued ligation of 10-kb DNA to high molecular weight DNA. Furthermore, using sequential treatment with aphidicolin and 3-aminobenzamide, one can observe the conversion of radiolabeled Okazaki fragments into 10-kb intermediates. The 10-kb DNA pieces are, however, not ligated to high molecular weight DNA in the presence of 3-aminobenzamide. Our results imply that functioning poly(ADP-ribose) synthetase is necessary for the ligation process.**

Eukaryotic DNA synthesis is very complex and there exist several steps in the synthetic pathway that are not understood (1). For example, we know very little about the steps in the maturation of primary DNA replication intermediates (Okazaki fragments) to high molecular weight DNA. Our inability to examine this process has been due in part to the large size of the eukaryotic genome, which has made it difficult to detect defined DNA replication intermediates that are larger than Okazaki fragments.

We have examined the ligation of a large DNA replication intermediate. Using a novel procedure to lyse cells in dilute alkali, we have detected at least two discrete DNA replication intermediates, Okazaki fragments and 10-kilobase (kb) fragments (2–4). The formation of both the Okazaki fragments and 10-kb DNA replication intermediates is dependent on DNA polymerase α (3). However, the ligation of 10-kb DNA to high molecular weight DNA still occurs when DNA polymerase α is inhibited by aphidicolin.

Poly(ADP-ribose) synthetase catalyzes the poly(ADP-ribosyl)ation of chromosomal proteins (5–8). Histones, mainly histone H1, are the major acceptors. Much work has been performed in attempts to understand the biological significance of this secondary modification. It is known that the poly(ADP-ribosyl)ation of histones results in relaxation of polynucleosomes (9, 10) and that the activity of poly(ADP-ribose) synthetase is dependent on nicks in the DNA (5–8, 11). In cells synchronized with a G_1 block, poly(ADP-ribosyl)ation exhibits a cell cycle-dependent oscillation with a maximum coinciding with S phase (12). This observation suggests connection between poly(ADP-ribose) synthetase and S-phase events, such as DNA synthesis.

Poly(ADP-ribose) synthetase can be inhibited using the drug 3-aminobenzamide (13), which is a competitive inhibitor that appears to be highly specific. It inhibits the repair of DNA damage induced by various alkylating agents or by γ-irradiation. Previous reports suggest that it is the ligation of DNA during the repair process that is impaired (14–17). In agreement with this suggestion, it has recently been shown that alteration of chromatin structure by poly(ADP-ribose) synthetase influences the activity of the DNA ligases (18).

We have analyzed DNA synthesis in cultured human melanoma cells. We describe results showing a reduced ligation of the 10-kb DNA replication intermediate in cells treated with 3-aminobenzamide.

MATERIALS AND METHODS

Cells, Culture Methods, and Labeling with [^{3}H]Thymidine. A human melanoma cell line (CRL 1424), obtained from Flow Laboratories, was grown as monolayers at 37°C in 5% CO_2 in air. The culture medium was minimal essential medium (Eagle) with Earle's salt mixture, 2 mM L-glutamine, 10% fetal calf serum, and antibiotics. The culture medium routinely was changed twice weekly and the cells were passaged every 4–6 days. For experiments, the cells were seeded in small culture dishes (35 × 10 mm), containing 3 ml of medium, 24 hr before the addition of drugs and [^{3}H]thymidine (24 Ci/mmol, 1 Ci = 37 GBq; Amersham).

Cell Lysis. The incubation medium was drained from the culture dish and the cells were rinsed twice in cold phosphate-buffered saline. Cells were lysed in the dark at 0°C in 2.25 ml of 0.03 M NaOH. After 30 min, the solution was neutralized by the addition of 0.9 ml of 0.067 M HCl/0.02 M NaH_2PO_4. Finally, the solution was made 0.5% in $NaDodSO_4$.

In experiments involving CsCl gradient centrifugations, the sample was sonicated, after the neutralization step, for 2 × 15 sec with a Branson sonifier equipped with a microtip and set at maximum level. The sample was then made 0.5% in *N*-lauroyl sarcosine.

Gel Electrophoresis. The 0.75% agarose flat-bed gels were made as described (19). Labeled DNA was separated in the agarose gels using an LKB Multiphor electrophoretic system. DNAs of known molecular weight, used as markers, were obtained from New England Nuclear. The gels were cut into 1-mm-thick slices that were assayed for radioactivity, using a toluene-based scintillation fluid containing 3% Soluene 100, in a Packard scintillation counter.

CsCl Gradient Centrifugation. CsCl was added to solutions of DNA in 0.01 M Tris Cl, pH 8/1 mM EDTA to give a refractive index of 1.3990. Gradient centrifugations were at 38,000 rpm at 23°C for 40 hr in a Ti50 rotor in a Beckman L2-65B ultracentrifuge. After centrifugation, the gradients were divided into 30–40 fractions, and the labeled DNA in each fraction was precipitated with trichloroacetic acid and collected on Whatman GF/C filters. Radioactivity on the filters was measured using a Packard scintillation counter.

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Abbreviation: kb, kilobase(s).

RESULTS

To release DNA replication intermediates from bulk DNA, we lyse cells in dilute alkali (2–4). The treatment with alkali denatures the DNA and initiates uncoiling of the DNA strands. This results in release of single-stranded DNA replication intermediates from bulk DNA, which are then separated by agarose gel electrophoresis. The uncoiling is initiated at gaps (or alkali-labile regions) in the DNA. Gaps can be induced in the DNA by γ-irradiation.

In undisturbed cells, there are gaps at the replication forks that serve as initiation points for uncoiling. Furthermore, gaps (or alkali-labile regions) exist at ≈10-kb intervals in newly synthesized DNA before it has matured to high molecular weight DNA (2, 3).

The high molecular weight DNA, in contrast to the replication intermediates, does not completely uncoil in the alkali. Therefore, it renatures when the solution is neutralized and it appears as double-stranded DNA on CsCl gradients. The experimental approach, which can be visualized as a selective melting of the replicon, facilitates the characterization of DNA replication intermediates.

Increased Amounts of Single-Stranded DNA in Cells Treated with 3-Aminobenzamide. First, we examined the banding properties in CsCl gradients of DNA labeled in cells incubated in the presence of 3-aminobenzamide (3 mM) for 60 min, with [^{3}H]thymidine present during the last 30 min. The cells were lysed in dilute alkali and sonicated, and the DNA was then banded in neutral CsCl gradients. Most of the radioactivity was in single-stranded DNA, while only a small amount of the label appeared in the double-stranded region (Fig. 1). In control cells, not incubated with 3-aminobenza-

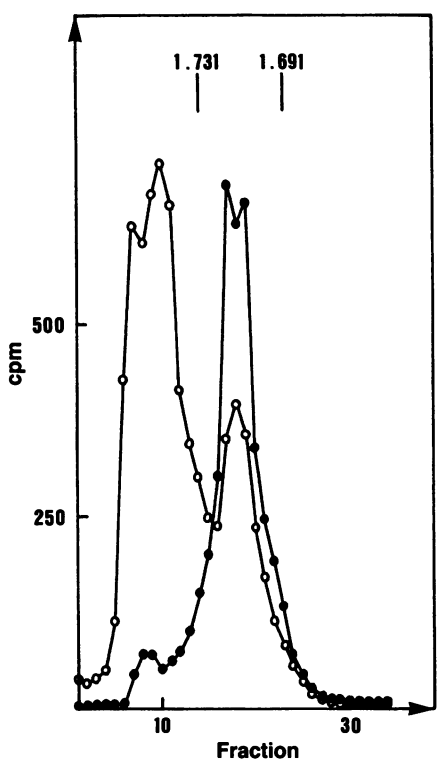


FIG. 1. Neutral CsCl-gradient separations of radiolabeled DNA from cells incubated with 3-aminobenzamide (3 mM) for 60 min with [^{3}H]thymidine present during the last 30 min of the drug treatment (○) and from control cells incubated with thymidine for 30 min (●). The cells were lysed in dilute alkali, the solution was neutralized, and detergent (*N*-lauroyl sarcosine) was then added. The material located at fractions 6–11 contains single-stranded DNA whereas the material located at fractions 15–21 contains double-stranded DNA. The locations of marker DNAs of known densities (1.731 and 1.691) are indicated.

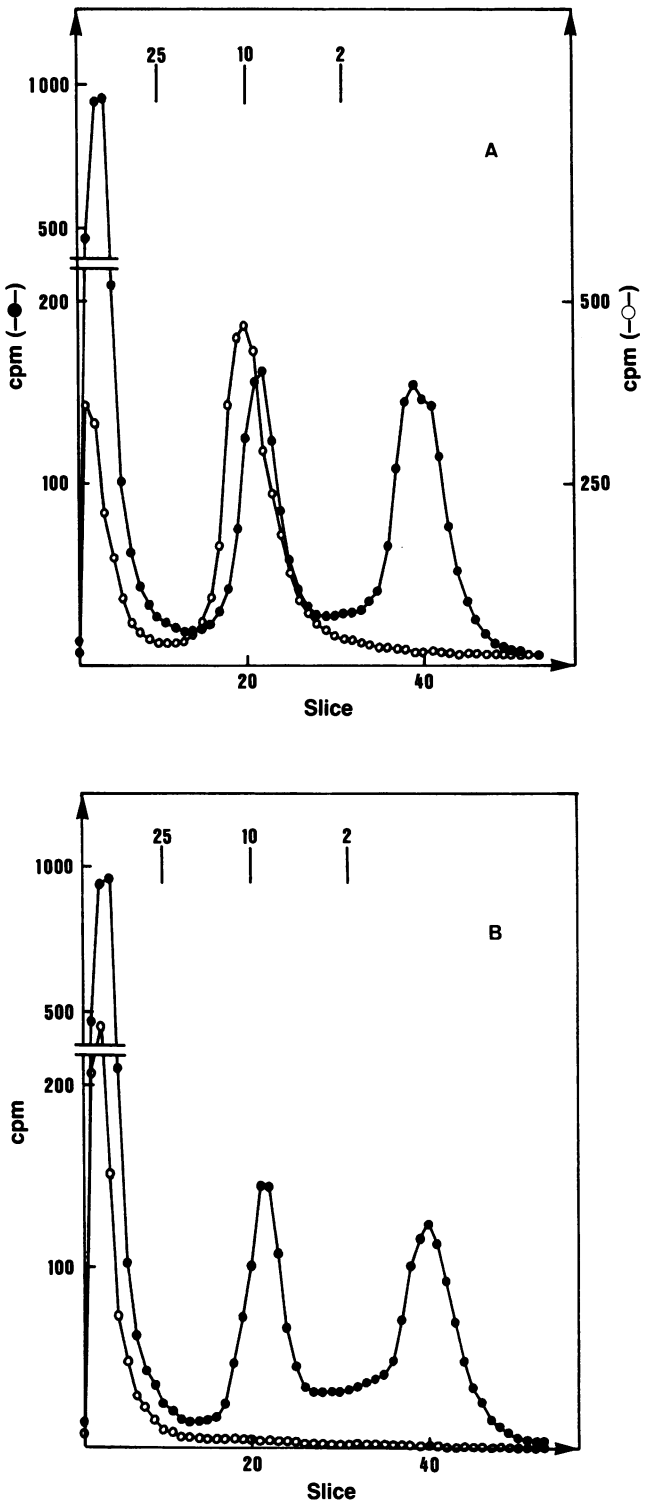
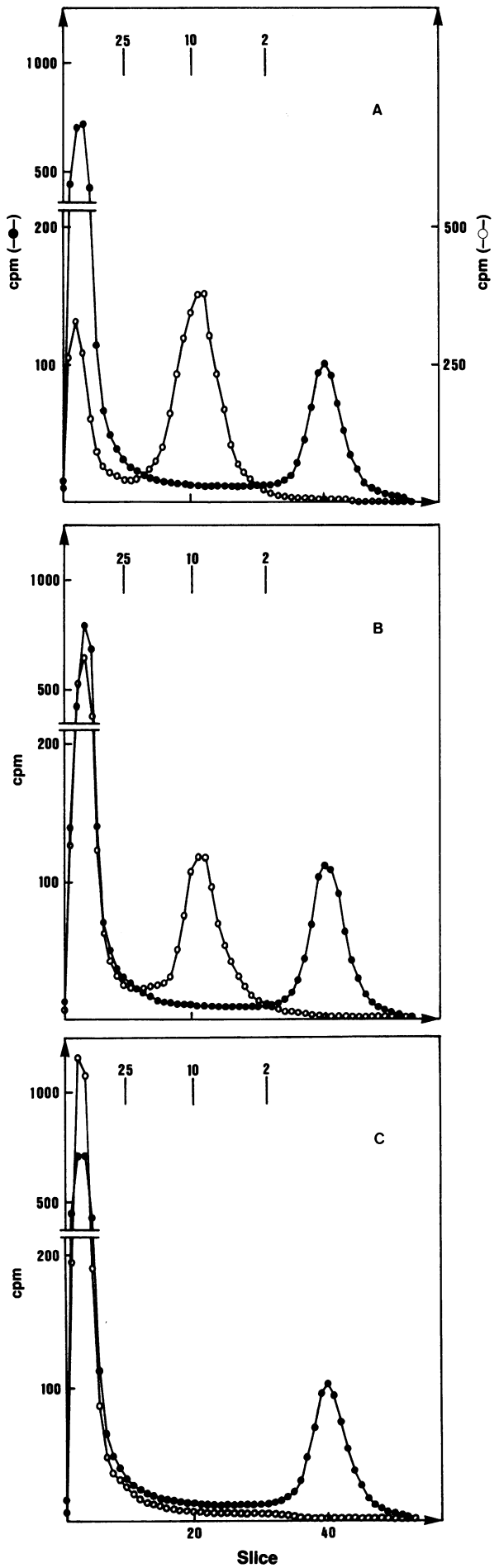


FIG. 2. (*A*) Gel electrophoretic separations of labeled DNA from cells incubated sequentially with 3-aminobenzamide (3 mM) for 30 min, [^{3}H]thymidine and 3-aminobenzamide for 5 min, and 3-aminobenzamide for 30 min (○) and from control cells incubated with [^{3}H]thymidine for 5 min (●). (*B*) Gel electrophoretic separations of labeled DNA from cells incubated with aphidicolin (10 μg/ml) for 30 min and then with [^{3}H]thymidine and aphidicolin for another 5 min (○) and from control cells incubated with [^{3}H]thymidine for 5 min (●). In all cases, cells were lysed in dilute alkali, the solution was neutralized, and detergent ($NaDodSO_4$) was added. The separation was performed in 0.75% agarose gels. Numerals across the top (25, 10, and 2) denote the location and size (in kb) of single-stranded DNA markers. The 10-kb DNA intermediate is located at slices 17–22, the Okazaki fragments at slices 35–41, and the double-stranded high molecular weight DNA at slices 3–6.



mide, most of the label appeared in double-stranded DNA. There was only slightly less total radioactivity incorporated into DNA in cells treated with 3-aminobenzamide than in control cells, in agreement with the findings of others (16, 17). Thus, although there is no or very little reduction in total incorporation of label into DNA, there is a large amount of single-stranded DNA fragments present in cells treated with 3-aminobenzamide. In untreated cells, most of the labeled DNA is double-stranded. The results indicate that, in the presence of the inhibitor, there is reduced ligation of DNA replication intermediates.

Accumulation of 10-kb DNA Replication Intermediates in Cells Treated with 3-Aminobenzamide But Not in Cells Treated with Aphidicolin. We have previously described (2, 3) a single-stranded 10-kb DNA replication intermediate. The 10-kb DNA has a longer half-life than Okazaki fragments. The synthesis of 10-kb DNA is dependent on DNA polymerase α, while the ligation of existing 10-kb DNA to high molecular weight DNA occurs independent of DNA polymerase α. A prerequisite for the existence of 10-kb DNA is the presence of gaps (or alkali-labile regions) located about 10-kb apart in the newly synthesized DNA (2, 3).

We examined the effect of 3-aminobenzamide on the joining of 10-kb DNA replication intermediates. Cells were preincubated with 3-aminobenzamide for 30 min, incubated with [^{3}H]thymidine for 5 min in the presence of the drug, and then incubated without label for another 30 min in the presence of the drug. The cells were lysed in dilute alkali, and the DNA was separated by agarose gel electrophoresis. There was an increased amount of labeled 10-kb DNA present in the treated cells (Fig. 2*A*). This was paralleled by a reduction in the amount of labeled high molecular weight DNA.

In contrast, Fig. 2*B* shows that, in cells incubated with aphidicolin (10 μg/ml) for 30 min and then labeled with [^{3}H]thymidine for 5 min in the presence of that drug, there was no detectable 10-kb DNA. Aphidicolin is a specific inhibitor of DNA polymerase α, the enzyme responsible for the movement of the replication fork (20).

Hence, there is an accumulation of 10-kb DNA in cells treated with 3-aminobenzamide, but not in cells treated with aphidicolin. A probable interpretation is that 3-aminobenzamide reduces the ligation of 10-kb DNA into high molecular weight DNA, a process that is not impaired by aphidicolin.

Sequential Treatment with Aphidicolin and 3-Aminobenzamide Results in Transfer of Label from Okazaki Fragments to 10-kb DNA, But Not to High Molecular Weight DNA. If the above interpretation is correct, one should be able to observe the conversion of labeled Okazaki fragments to 10-kb DNA and then prevent the incorporation of 10-kb DNA into high molecular weight DNA, by using sequential treatments with aphidicolin and 3-aminobenzamide.

Therefore, cells were labeled with [^{3}H]thymidine for 5 min and then treated with aphidicolin for 30 min. From these cells, we detected labeled high molecular weight DNA and Okazaki fragments, but not labeled 10-kb DNA (3) (Fig. 3*A*). We simultaneously analyzed DNA from cells treated with 3-aminobenzamide as described in the section above. In the 3-

FIG. 3. Electrophoretic separations of labeled DNA from cells incubated under various conditions: (*A*) Five minutes with [^{3}H]thymidine and then 30 min with aphidicolin (10 μg/ml) (●) or 30 min with 3-aminobenzamide (3 mM), 5 min with [^{3}H]thymidine and 3-aminobenzamide, and finally 30 min with 3-aminobenzamide (the same protocol as in the legend to Fig. 2*A*) (○). (*B*) Five minutes with [^{3}H]thymidine, 30 min with aphidicolin, and finally 30 min with 3-aminobenzamide (○) or 5 min with [^{3}H]thymidine and then 30 min with aphidicolin (●). (*C*) Five minutes with [^{3}H]thymidine, then 30 min with aphidicolin, and finally 30 min in fresh medium (○) or 5 min with [^{3}H]thymidine and then 30 min with aphidicolin (●). Cell lysis and DNA electrophoresis are described in *Materials and Methods*. Markers and peak identities are described in the legend to Fig. 2.

aminobenzamide-treated cells there was a discrete 10-kb DNA population that was not present in the aphidicolin-treated cells.

Next, cells were labeled with [^{3}H]thymidine for 5 min, incubated with aphidicolin for 30 min, and then incubated in the presence of 3-aminobenzamide for another 30 min (Fig. 3*B*). A second set of cells were treated similarly, except that they were incubated in fresh medium during the last 30 min instead of in medium containing 3-aminobenzamide (Fig. 3*C*). When the DNA from these two sets of cells was analyzed by electrophoresis in agarose gels, there was a distinct peak of labeled 10-kb DNA in the DNA from cells post-incubated with 3-aminobenzamide. There was no such peak of labeled DNA from cells post-incubated in fresh medium.

Hence, labeled Okazaki fragments are incorporated into 10-kb DNA but the 10-kb DNA is not ligated to high molecular weight DNA in the presence of 3-aminobenzamide.

The 10-kb DNA Is Ligated to High Molecular Weight DNA After Removal of 3-Aminobenzamide. To examine whether the 10-kb DNA intermediates that accumulate in cells treated with 3-aminobenzamide can form high molecular weight DNA, the following experiment was performed. Cells were incubated with 3-aminobenzamide for 30 min, with [^{3}H]thymidine and 3-aminobenzamide for 5 min, and then with 3-aminobenzamide for another 30 min (the protocol that results in accumulation of 10-kb intermediates, see Fig. 2*A*). The cells were then washed to remove the drug and incubated for another 3 hr in fresh medium. When the labeled DNA was analyzed, the gel electrophoretic separation showed only high molecular weight DNA (Fig. 4*A*) and the neutral CsCl gradient contained mainly double-stranded DNA (Fig. 4*B*). Therefore, the 10-kb DNA intermediates formed in the presence of 3-aminobenzamide do have the capability to mature to high molecular weight DNA after the drug is removed from the cells.

DISCUSSION

Poly(ADP-ribose) synthetase is a DNA-dependent chromatin-bound enzyme (5, 6). Its activity is enhanced by the presence of gaps or nicks in DNA. It is known that poly(ADP-ribosyl)ation of proteins during DNA repair occurs in the regions of the chromatin containing single-stranded DNA breaks.

During DNA replication there exist gaps at the replication forks. Gaps also exist at 10-kb intervals in the newly synthesized DNA. This may correlate with the earlier finding that there is increased poly(ADP-ribosyl)ation during S phase (12), since the presence of gaps in the DNA during replication should increase the enzymatic activity.

The primary DNA replication intermediates are formed at the replication fork. Using a new approach to release DNA replication intermediates from bulk DNA (cell lysis in dilute alkali), we showed previously that the primary pieces (Okazaki fragments) give rise to secondary pieces, 10-kb replication intermediates (2, 3). The joining of the 10-kb DNA to high molecular weight DNA occurs outside of the replication fork and does not involve DNA polymerase α. We have now used the drug 3-aminobenzamide to investigate that joining process.

The results show that 3-aminobenzamide reduces the ligation of 10-kb DNA. Cells pretreated with the drug contain large amounts of single-stranded 10-kb DNA. Furthermore, using sequential treatment with aphidicolin followed by 3-aminobenzamide, we observed the conversion of labeled Okazaki fragments into 10-kb DNA, but there was reduced ligation of 10-kb DNA into high molecular weight DNA. However, when the cells were washed free of the drug, there was resumed ligation of 10-kb DNA, generating high molecular weight DNA.

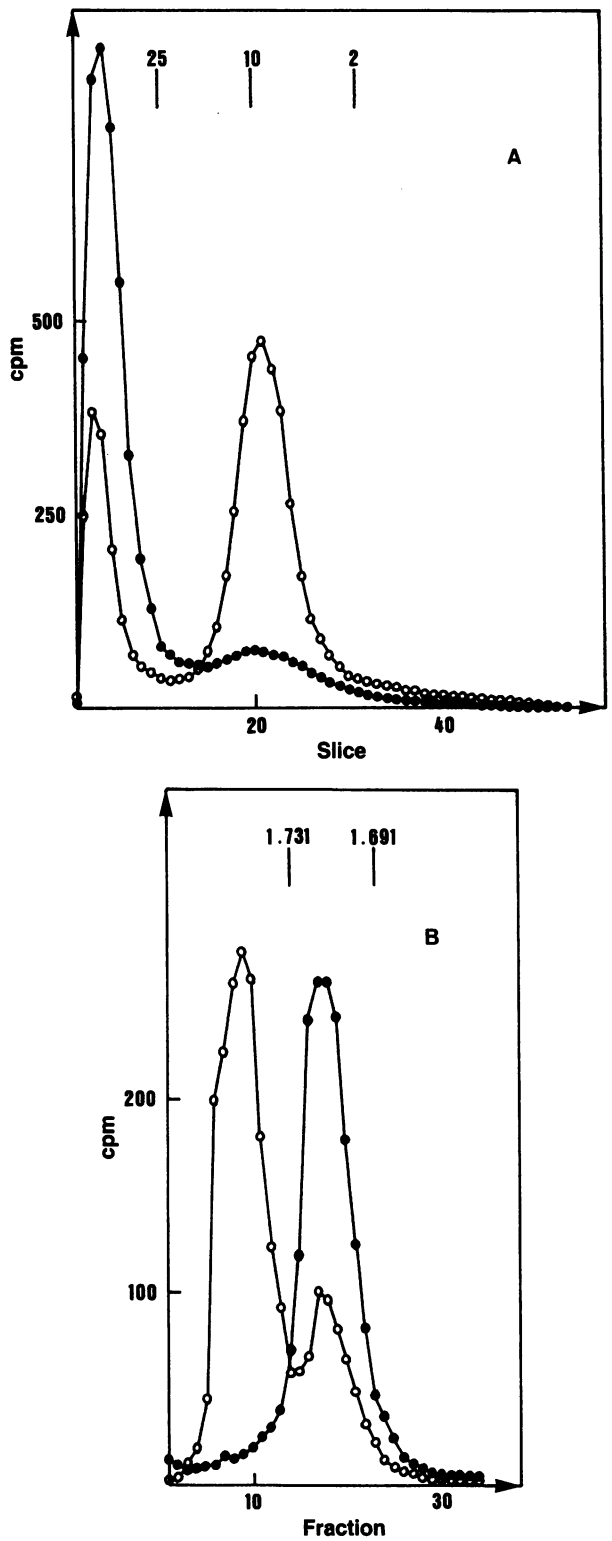


FIG. 4. (*A*) Electrophoretic separations of labeled DNA from cells incubated sequentially for 30 min with 3-aminobenzamide (3 mM), 5 min with [^{3}H]thymidine and 3-aminobenzamide, 30 min with 3-aminobenzamide, and finally for 3 hr in fresh medium (●) and from cells incubated similarly except for the 3 hr post-incubation in fresh medium (i.e., the same treatment protocol given in the legend to Fig. 2*A*) (○). Markers and identity of peaks are described in the legend to Fig. 2. (*B*) Neutral CsCl-gradient separations of labeled DNA from cells incubated as described in *A*. Markers and the locations of single- and double-stranded DNA are described in the legend to Fig. 1.

Taken together, the present results and our previous findings with aphidicolin (3) suggest the following: (*i*) The formation of Okazaki fragments and 10-kb DNA is dependent on

DNA polymerase α. (*ii*) The ligation of 10-kb DNA to high molecular weight DNA occurs by a mechanism not connected with the events at the replication fork. (*iii*) The ligation of 10-kb DNA is slowed by treatment with 3-aminobenzamide, resulting in an accumulation of 10-kb DNA replication intermediates. (*iv*) The enzyme poly(ADP-ribose) synthetase is involved in the ligation of 10-kb DNA.

Poly(ADP-ribose) synthetase is believed to be located mainly in the internucleosomal regions of chromatin (21, 22). The enzyme has the capacity for extensive self-modification and effects poly(ADP-ribosyl)ation of various chromatin components (4, 5), especially histones (mainly H1). Polynucleosomes are fully relaxed when they are poly(ADP-ribosyl)ated (9, 10), and it is known that poly(ADP-ribosyl)ation of isolated chromatin increases DNA ligase activity (18). Therefore, the existence of poly(ADP-ribosyl)ated relaxed polynucleosomes may favor the ligation steps of DNA replication. 3-Aminobenzamide prevents the poly(ADP-ribosyl)ation and, therefore, probably reduces DNA ligation. Such a mechanism may explain the reduced ligation of 10-kb DNA replication intermediates in 3-aminobenzamide-treated cells.

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1. Becker, Y., ed. (1983) *Molecular Events in the Replication of Viral and Cellular Genomes* (Nijhoff, The Hague, Netherlands).
2. Lönn, U. (1982) *Chromosoma* **84**, 663–673.
3. Lönn, U. & Lönn, S. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3996–3999.
4. Ahnström, G. & Erixon, K. (1981) in *DNA Repair, A Laboratory Manual of Research Procedures*, eds. Friedberg, E. & Hanawalt, P. (Dekker, New York), Part B, pp. 403–418.
5. Hayaishi, O. & Ueda, K. (1977) *Annu. Rev. Biochem.* **46**, 95–116.
6. Smulson, M. E. & Sugimura, T. (1980) *Novel ADP-Ribosylations of Regulatory Enzymes and Proteins* (Elsevier/North-Holland, New York).
7. Purnell, M., Stone, P. & Whish, W. (1980) *Biochem. Soc. Trans.* **8**, 215–227.
8. Ueda, K., Omachi, A., Kawaichi, M. & Hayaishi, O. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 205–209.
9. Poirier, G. G., DeMurcia, G., Jongstra-Bilen, J., Neidergang, C. & Mandel, P. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3423–3429.
10. Aubin, R. J., Frechette, A., DeMurcia, G., Mandel, P., Lord, A., Grandin, G. & Poirier, G. G. (1983) *EMBO J.* **2**, 1685–1693.
11. Benjamin, R. C. & Gill, D. M. (1980) *J. Biol. Chem.* **255**, 10502–10508.
12. Kidwell, W. R. & Mage, M. G. (1976) *Biochemistry* **15**, 1213–1217.
13. Purnell, M. & Whish, W. (1980) *Biochem. J.* **185**, 775–777.
14. Durkacz, B., Omidiji, O., Gray, D. & Shall, S. (1980) *Nature (London)* **283**, 593–596.
15. Creissen, D. & Shall, S. (1982) *Nature (London)* **296**, 271–272.
16. James, M. & Lehman, A. (1982) *Biochemistry* **21**, 4007–4013.
17. Morgan, W. & Cleaver, J. (1983) *Cancer Res.* **43**, 3104–3107.
18. Ohashi, Y., Ueda, K., Kawachi, M. & Hayaishi, O. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3604–3607.
19. Lönn, U. (1978) *Cell* **13**, 727–733.
20. Huberman, J. (1981) *Cell* **23**, 647–648.
21. Giri, C. P., West, M. H. P., Davies, M. L. & Smulson, M. E. (1978) *Biochemistry* **19**, 3501–3504.
22. Mullins, D. W., Giri, C. P. & Smulson, M. E. (1977) *Biochemistry* **16**, 506–513.